



Atty. Docket No.: 3284/1235

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Habener et al.
Serial No.:	09/963,875
Filed:	September 26, 2001
Titled:	Stem Cells of the Islets of Langerhans and Their Use In Treating Diabetes Mellitus

Examiner: M.A. Belyavskyi

Group Art Unit: 1644

Conf. No.: 9674

DECLARATION UNDER 37 CFR 1.132 BY JOEL F. HABENER, M.D.

I declare:

1. I, Joel F. Habener hold an M.D. degree from the University of California, Los Angeles. I received my M.D. degree in 1965. My current positions are Investigator, Howard Hughes Medical Institute at the Massachusetts General Hospital, Associate Physician at the Massachusetts General Hospital and Professor of Medicine at Harvard Medical School. I have held the position of Howard Hughes Investigator since 1976. I have held the position of Associate Physician since 1989. I have held the position of Professor of Medicine at Harvard Medical School since 1989. Previously, I held the position of Associate Professor of Medicine at Harvard Medical School from 1975-1988. I am an inventor of the above-referenced patent application.
2. I have read the Office Action dated December 23, 2003, filed in the above-referenced patent application.
3. My laboratory has developed a method for isolating a stem cell from a pancreatic islet of

Langerhans that includes the steps of: (a) removing a pancreatic islet from a donor, (b) removing cells from said pancreatic islet wherein said islet comprises a plurality of cell types comprising stem cells; and (c) separating said stem cells from said plurality of cells.

4. Isolated human pancreatic islets were obtained through the JDRF Human Islet Distribution Program from the following centers: The Joslin Diabetes Center, Boston, the Northwest Tissue Center Islet and Cell Processing Laboratory, Seattle, and the Islet Distribution Center at the Diabetes Research Institute, Miami. Culture conditions were as follows. Single cell suspensions of Human pancreatic islet preparations were made by digestion with 5mg/ml Trypsin in PBS at 37°C and passage through a glass pipette. Viable cells were counted by Trypan Blue exclusion and seeded at 10,000 cells/cm² on tissue culture treated plastic dishes (Corning, Corning, NY). In initial experiments cell expansion was done in RPMI 1640 (11 mmol/l Glucose) (Invitrogen, Carlsbad, CA) with 10 mmol/l Hepes buffer, 1 mmol/l sodium pyruvate, 10% fetal bovine serum (FBS; Omega Scientific, Tarzana, CA), 25ng/ml EGF, 20ng/ml bFGF and 1x penicillin/streptomycin. In later experiments CMRL 1066 medium (5.5 mmol/l Glucose) with 10% FBS, 1x penicillin/streptomycin, 100ng/ml beta nerve growth factor (β-NGF; R&D Systems, Minneapolis, MN), and 25ng/ml EGF was used. 24 to 48 hours after seeding, dead cells were removed by a media change and one wash with PBS. Thereafter, cells were expanded for 10-14 days until they reached confluence and medium was changed every 3 days.

According to this protocol, upon culture initiation only a few cells (less than 10%) attached to the dish and began to proliferate whereas the majority of cells did not attach to the dish and were washed off and discarded with the subsequent 2-3 changes of the culture media. Over 10-14 days of culture, the cells reached confluency. At this time, the levels of insulin mRNA in the cultured cells had markedly decreased compared to that of initial islets.

Immunostaining revealed only an occasional insulin-positive cell within the monolayer of expanded cells.

The resulting expansion cultures of progenitor cells contain at least two phenotypically distinct cell types, those that express nestin and vimentin and those that express epithelial markers cytokeratin 19 and E-cadherin, as detected by immunofluorescent staining as described below.

Expansion phase cells were grown on tissue culture treated plastic slides (Nalge Nunc, Naperville, IL). For immunostaining cells were fixed with PBS/4% paraformaldehyde for 10 minutes at room temperature (RT). Slides were blocked with normal donkey serum in PBS/0.1%Triton for 1 hour at RT, incubated with primary antibodies over night at 4°C, washed, incubated with Cy2/Cy3 labeled secondary antibodies for 1 hour at RT, washed again and mounted. In vitro generated cell clusters were embedded in a fibrin clot prior to fixation. Clotting was achieved by mixing solutions of human fibrinogen (80 mg/ml in PBS, Sigma, St. Louis, MO) and human thrombin (50 units/ml in 40 mmol/l CaCl₂, Sigma). Clots were fixed in 10% buffered formalin, dehydrated, embedded in paraffin and cut into 4µm sections. Sections were dewaxed in xylene, hydrated, boiled for 10 minutes in a microwave oven in 10mmol/l sodium citrate, pH 6 for antigen retrieval and stained as described above. Nuclei were counterstained with DAPI. Antibodies used were guinea pig anti-insulin (1:2,000), guinea pig anti-glucagon (1:2,000), rabbit anti-somatostatin (1:2,000), and guinea pig anti-pancreatic polypeptide (1:2,000; all from Linco, St. Charles, MS), rabbit anti human nestin (1:200, Chemicon, Temecula, CA), mouse anti vimentin (1:100, Signet, Dedham, MA) mouse anti smooth muscle actin (1:100, Sigma), mouse anti cytokeratin 19 (1:100, Sigma), mouse anti keratin (1:100, Chemicon), mouse anti desmin (1:100, Sigma).

Immunostaining revealed that expansion cultures consisted of two major types of cells: E-cadherin/cytokeratin 19 (CK19) positive epithelial cells growing in patches and vimentin/nestin-positive spindle-shaped cells growing separately from each other. Many of the spindle-shaped cells also co-expressed smooth muscle actin. Occasional cells with epithelial characteristics (E-cadherin and CK19-positive) also stained positive for nestin (See Figure 1A-F, attached).

The two major populations of cells are easily separated based on differences in their

morphologies. The nestin/vimentin positive spindle shaped fibroblastoid cells are markedly different from that of the E-cadherin/CK19 positive flat, cuboidal epithelial-like cells that are in patches. Under regular or phase contrast light microscopy, using low power, nestin/vimentin positive cells that are clearly separated from the E-cadherin/CK19 cells which are in distinct patches are selected. In certain embodiments, the nestin/vimentin positive cells are "cloned" by replating them and expanding them, multiple times.

The majority of the spindle shaped cells are nestin and vimentin positive as demonstrated by repeated immunohistochemical staining of expansion phase cultures.

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Antibodies used were guinea pig anti-insulin (1:2,000), guinea pig anti-glucagon (1:2,000), rabbit anti-somatostatin (1:2,000), and guinea pig anti-pancreatic polypeptide (1:2,000; all from Linco, St. Charles, MS), rabbit anti human nestin (1:200, Chemicon, Temecula, CA), mouse anti vimentin (1:100, Signet, Dedham, MA) mouse anti smooth muscle actin (1:100, Sigma), mouse anti cytokeratin 19 (1:100, Sigma), mouse anti keratin (1:100, Chemicon), mouse anti desmin (1:100, Sigma).

5. In view of the following, I believe that 0.2 to 5% of the cells of the pancreas are nestin-

positive cells.

To estimate the number of nestin-positive cells in a pancreas my laboratory overlaid a transparent grid over Figures 1D and 6C of Zulewski, H., Abraham, EJ, Gerlach, MJ, Daniel, PB, Moritz, W., Muller, B., Vallejo, M., Thomas, MK, and Habener, JF, *Diabetes*, 2001, 50:521-533, counted the nestin positive cells (yellow) and divided by the total number of DAPI positive cells (blue). Using this methodology we calculated that approximately 10% of the cells are nestin positive. However, these figures depict highly selected fields chosen to maximize the number of nestin-positive cells in the figures. The section of the islet shown in Figure 1D has 25 nestin positive cells. Since some islets have one or two nestin positive cells while other islets have as many as 10-20 nestin positive cells, I estimate that the number of nestin-positive cells in the islets is an average of 5-10 cells or 2-4%, on average. Nestin- positive cells tend to gather in islets and around ducts. Since only 2-3% of the cells of the entire pancreas are islet and ducts, I estimate that based on the results of the above analysis, 0.2 to 0.4% of the total cells of the pancreas are nestin positive.

To estimate the number of nestin-positive cells in the pancreas my laboratory performed immunocytochemistries on pancreas sections (nestin antiserum diluted 1:500). The number of nestin-positive cells in four fields containing non-islet cells is 26 of 461 DAPI-stained nuclei (5.6%), 23 of 442 DAPI-stained nuclei (5.2%), 14 of 408 DAPI-stained nuclei (3.4%) and 12 of 428 DAPI-stained nuclei (2.8%). The contribution of nestin-positive cells from the non-islet cells of the pancreas is therefore 4.25%, the average value for these four fields. The number of nestin-positive cells in four fields

containing only islets (insulin staining was used to identify the islet borders) is 19 of 375 DAPI-stained nuclei (5.1%), 21 of 234 DAPI-stained nuclei (9.0%), 15 of 243 DAPI-stained nuclei (6.1%) and 6 of 132 DAPI-stained nuclei (4.58%). The average value for these four fields is 6.2%. The islets make up approximately 2% of the total pancreas tissue. The contribution of nestin positive cells from the islets is 2% of 6.2% or 0.124%. In view of the above, the total number of nestin-positive cells, as determined by the immunocytochemical method described above, is approximately 4.4%.

It is my belief that in view of the above, one of skill in the art would accept that the percentage of nestin-positive cells in the pancreas is in the range of 0.2 to 5%, as determined by the two independent methods described hereinabove.

I hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

June 17, 2004
Date

Joel F. Habener
Joel F. Habener